PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION 09/480,251

International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International patent classification[6]: C12N 15/82, 15/62, C07K 14/435, C12N 15/12, A01H 5/00, A01N 63/00 — A1

(11) International publication number: WO 99/02717

(43) International publication date: 21 January 1999 (21.01.99)

(21) International application number: PCT/FR98/01462

(22) International filing date: 8 July 1998 (08.07.98)

(30) Data relating to the priority:
97/09,115 11 July 1997 (11.07.97) FR
97/09,663 24 July 1997 (24.07.97) FR

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(81) Designated states: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With the International Search Report.
Before expiry of the period provided for amending the claims, will be republished if such amendments are received.

As printed

(54) Title: CHIMERIC GENE CODING FOR DROSOMICINE, VECTOR CONTAINING IT AND PRODUCTION OF TRANSGENIC PLANTS RESISTANT TO DISEASES

(54) Titre: GENE CHIMERE CODANT POUR LA DROSOMICINE, VECTEUR LE CONTENANT ET OBTENTION DES PLANTES TRANSGENIQUES RESISTANTES AUX MALADIES

(57) Abstract

The invention concerns a chimeric gene containing a DNA sequence coding for drosomicine, a vector containing the chimeric gene, and a method for transforming plants and the resulting transformed plants. The drosomicine produced by the plants provides them with resistance to diseases, in particular of fungal origin.

(57) Abrégé

La présente invention a pour objet un gène chimère contenant une séquence d'ADN codant pour la drosomicine, un vecteur contenant le gene chimère, un procédé pour la transformation des plantes et les plantes transformées. La drosomicine produite par les plantes tranformées leur confère une résistance aux maladies, en particulier d'origine fongique.

CHIMERIC GENE ENCODING DROSOMYCIN, VECTOR CONTAINING IT AND PRODUCTION OF DISEASE-RESISTANT TRANSGENIC PLANTS

The subject of the present invention is a DNA sequence encoding drosomycin, a chimeric gene containing it, a vector containing the chimeric gene and a method of transforming plants and the disease-resistant transformed plants.

There is nowadays an increasing need to make plants resistant to diseases, in particular fungal diseases, in order to reduce or even avoid the need to resort to treatments with antifungal protection products, so as to protect the environment. One way of increasing this resistance to diseases consists in transforming plants so that they produce substances capable of providing their defence against these diseases.

Various substances of natural origin, in particular peptides, are known which have bactericidal or fungicidal properties, in particular against fungi responsible for plant diseases. However, the problem consists in finding such substances which not only can be produced by transformed plants, but can still preserve their bactericidal or fungicidal properties and confer them on the said plants. For the purposes of the present invention, bactericide or fungicide is understood to mean both the actual bactericidal or

fungicidal properties and the bacteriostatic or fungistatic properties.

Drosomycins are peptides produced by the larvae and adults of drosophila by induction following septic injury or the injection of a low dose of bacteria. A peptide has already been described as having certain antifungal and antibacterial properties in vitro, in particular in French patent application 2,725,992, where the peptide is obtained by induction in drosophila and purification. The gene encoding this same peptide has also been described by Fehlbaum et al. (1994). The possibility of integrating this gene into a plant to confer on it resistance to diseases of fungal or bacterial origin has, however, so far not been described.

It has now been found that the genes for drosomycins could be inserted into plants to confer on them properties of resistance to fungal diseases and to diseases of bacterial origin, providing a particularly advantageous solution to the problem set out above.

The subject of the invention is therefore firstly a chimeric gene comprising a nucleic acid fragment encoding a drosomycin as well as heterologous regulatory elements at positions 5' and 3' capable of functioning in plants and a vector for the transformation of plants containing this chimeric gene. It also comprises a transformed plant cell containing at least one nucleic acid fragment encoding drosomycin

and a disease-resistant plant containing the said cell. It finally relates to a method of transforming plants to make them resistant to diseases, in which a gene encoding drosomycin is inserted.

Drosomycin is understood to mean according to the invention any peptide capable of being isolated from the larvae and adults of drosophila by induction following a septic injury or the injection of a low dose of bacteria, these peptides comprising at least 44 amino acids and 8 cystein residues forming disulphide bridges with each other.

Advantageously, drosomycin essentially comprises the peptide sequence of formula (I) below:

Xaa-Cys-Xab-Cys-Xac-Cys-Xad-Cys-
Xae-Cys-Xaf-Cys-Xag-Cys-Xah-Cys (I)

in which

Xaa represents a peptide residue comprising at least 1 amino acid,

Xab represents a peptide residue of 8 amino acids,

Xac represents a peptide residue of 7 amino acids,

Xad represents a peptide residue of 3 amino acids,

Xae represents a peptide residue of 9 amino acids,

Xaf represents a peptide residue of 5 amino acids,

Xag represents a peptide residue of one amino acid, and

Xah represents a peptide residue of 2 amino acids.

Advantageously, Xab and/or Xad and/or Xac comprise at least one basic amino acid. More advantageously, Xab comprises at least 2 basic amino acids, preferably 2 and/or Xad and/or Xaf comprise at least 1 basic amino acid, preferably 1. Basic amino acid is understood to mean according to the invention the amino acids chosen from lysine, arginine or homoarginine.

Preferably,

Xaa represents the peptide sequence Xaa'-Asp- in which Xaa' represents NH_2 or a peptide residue comprising at least 1 amino acid, and/or

Xab represents the peptide sequence -Leu-Xab'-Pro- in which Xab' represents a peptide residue of 6 amino acids, and/or

Xac represents the peptide sequence -Ala-Xac'-Thr- in which Xac' represents a peptide residue of 5 amino acids, and/or

Xad represents the peptide sequence -Arg-Xad'-Val, in which Xad' represents a peptide residue of one amino acid, and/or

Xae represents the peptide sequence -Lys-Xae'-His- in which Xae' represents a peptide residue of 7 amino acids, and/or

Xaf represents the peptide sequence -Ser-Xaf'-Lys- in which Xaf' represents a peptide residue of 3 amino acids, and/or

Xag represents Trp, and/or

Xah represents the peptide residue Glu-Gly.

Preferably,

Xab' represents the peptide sequence Ser-Gly-Arg-Tyr-Lys-Gly, and/or

Xac' represents the peptide sequence Val-Trp-Asp-Asn-Glu, and/or

Xad' represents Arg, and/or

Xae' represents the peptide sequence Glu-Glu-Gly-Arg-Ser-Ser-Gly, and/or

Xaf' represents the peptide sequence Pro-Ser-Leu.

According to a more preferred embodiment of the invention, drosomycin is the peptide sequence represented by the sequence identifier No. 4 (SEQ ID No. 4) and the homologous peptide sequences.

Homologous peptide sequences is understood to mean any equivalent sequence comprising at least 65% homology with the sequence represented by the sequence identifier No. 4, it being understood that the 8 cystein residues and the number of amino acids separating them remain identical, some amino acids being replaced with different but equivalent amino acids at sites which do not induce substantial modification of the antifungal or antibacterial activity of the said homologous sequence. Preferably, the homologous sequences comprise at least 75% homology, more preferably at least 85% homology, still more preferably 90% homology.

The terminal NH_2 residue may exhibit a post-translational modification, for example an acetylation, in the same way that the C-terminal residue may exhibit a post-translational modification, for example an amidation.

Peptide sequence mainly comprising the peptide sequence of general formula (I) is understood to mean not only the sequences defined above, but also such sequences comprising at either of their ends, or both, peptide residues, in particular which are necessary for their expression and targeting in plant cells or in plants.

It is in particular "full-length" drosomycin represented by the sequence identifier No. 2 (SEQ ID No. 2).

It is in particular a "peptide-drosomycin" or "drosomycin-peptide", advantageously "peptide-drosomycin" fusion peptide, the cutting of which by the enzymatic systems of plant cells allows the liberation of the drosomycin defined above. The peptide fused with drosomycin may be a signal peptide or a transit peptide which makes it possible to control and orient the production of drosomycin in a specific manner in a part of the plant cell or of the plant, such as for example the cytoplasm, the cell membrane, or in the case of plants in a specific type of cell compartment or of tissues or in the extracellular matrix.

According to one embodiment, the transit peptide may be a chloroplast or mitochondrial addressing signal, which is then cleaved in the chloroplasts or mitochondria.

According to another embodiment of the invention, the signal peptide may be an N-terminal or "prepeptide" signal, optionally in combination with a signal responsible for retaining the protein in the endoplasmic reticulum, or a vacuolar addressing peptide or "propeptide". The endoplasmic reticulum is the site where the "cellular machinery" carries out the operations of maturation of the protein produced, such as for example the cleavage of the signal peptide.

The transit peptides may be either single, or double, and in this case optionally separated by an intermediate sequence, that is to say comprising, in the direction of transcription, a sequence encoding a transit peptide for a plant gene encoding a plastid localization enzyme, a portion of sequence of the N-terminal mature portion of a plant gene encoding a plastid localization enzyme, and then a sequence encoding a second transit peptide for a plant gene encoding a plastid localization enzyme, as described in Patent EP 0 508 909.

As transit peptide useful according to the invention, there may be mentioned in particular the signal peptide of the tobacco PR-1α gene (WO 95/19443),

or alternatively the ubiquitin represented, fused with drosomycin by the sequence identifier No. 6.

The fusion peptide "ubiquitin-drosomycin" and its coding sequence are also included in the present invention, in particular described by the sequence identifier No. 5.

The present invention therefore relates to a chimeric gene comprising a sequence encoding drosomycin as well as heterologous regulatory elements at positions 5' and 3' capable of functioning in plants, the coding sequence comprising at least one DNA sequence encoding drosomycin as defined above.

The DNA sequence may be obtained according to standard methods of isolation and purification from drosophila, or alternatively by synthesis according to the usual techniques of successive hybridizations of synthetic oligonucleotides. These techniques are in particular described by Ausubel *et al*.

According to one embodiment of the invention, the DNA sequence encoding drosomycin comprises the DNA sequence described by bases 21 to 152 of the sequence identifier No. 3 (SEQ ID NO 3), a sequence homologous or complementary to the said sequence.

According to another embodiment of the invention, the DNA sequence encoding "full-length" drosomycin comprises the DNA sequence described by bases 101 to 310 of the sequence identifier No. 1 (SEQ

ID NO 1), a sequence homologous or complementary to the said sequence.

According to another embodiment of the invention, the DNA sequence encoding the "peptide-heliomycin" fusion peptide comprises the DNA sequence described by bases 15 to 221 of the sequence identifier No. 5 (SEQ ID NO 5), a homologous sequence or a sequence complementary to the said sequences.

"Homologous" is understood to mean according to the invention any DNA sequence having one or more sequence modifications compared with the nucleotide sequence described by the sequence identifiers No. 1, 3 or 5 and encoding drosomycin, "full-length" drosomycin or the "peptide-drosomycin" fusion peptide. These modifications may be obtained according to the usual mutation techniques, or alternatively by choosing the synthetic oligonucleotides used in the preparation of the said sequence by hybridization. In the light of the multiple combinations of nucleic acids which can lead to the expression of the same amino acid, the differences between the reference sequence described by the sequence identifiers No. 1, 3 or 5 and the corresponding homologue may be substantial, all the more so since they are small-sized DNA fragments which can be made by chemical synthesis. Advantageously, the degree of homology will be at least 70% relative to the reference sequence, preferably at least 80%, more preferably at least 90%. These modifications are

generally neutral, that is to say that they do not affect the primary sequence of the resulting drosomycin or fusion peptide.

"Plant cell" is understood to mean according to the invention any cell derived from a plant and capable of constituting undifferentiated tissues such as calli, differentiated tissues such as embryos, plant portions, plants or seeds.

"Plant" is understood to mean according to the invention any differentiated multicellular organism capable of photosynthesis, in particular monccotyledonous or dicotyledonous plants, more particularly crop plants intended or not as animal feed or for human consumption, such as maize, wheat, colza, soya bean, rice, sugar cane, beet, tobacco, cotton and the like.

The regulatory elements necessary for the expression of the DNA sequence encoding drosomycin are well known to persons skilled in the art according to the plant. They comprise in particular promoter sequences, transcription activators, terminator sequences, including start and stop codons. The means and methods for identifying and selecting the regulatory elements are well known to persons skilled in the art. As promoter regulatory sequence in plants, there may be used any promoter sequence of a gene which is naturally expressed in plants, in particular a promoter of bacterial, viral or plant origin such as,

for example, that of a ribulose-biscarboxylase/ oxygenase (RuBisCO) small subunit gene or of a plant virus gene such as, for example, that of the cauliflower mosaic (CAMV 19S or 35S), or a promoter inducible by pathogens such as tobacco PR-1a, it being possible to use any known suitable promoter. Preferably, a regulatory promoter sequence is used which promotes the overexpression of the coding sequence constitutively or inducibly upon attack by a pathogen, such as for example that comprising at least one histone promoter as described in application EP 0 507 698.

According to the invention, it is also possible to use, in combination with the regulatory promoter sequence, other regulatory sequences, which are situated between the promoter and the coding sequence, such as transcription enhancers, such as for example the translational enhancer of the tobacco mosaic virus (TMV) described in application WO 87/07644, or of the tobacco etch virus (TEV) described by Carrington & Freed.

As regulatory terminator or polyadenylation sequence, there may be used any corresponding sequence of bacterial origin, such as for example the *Agrobacterium tumefaciens* nos terminator, or alternatively of plant origin, such as for example a histone terminator as described in application EP 0,633,317.

The present invention also relates to an integrating vector for the transformation of plants containing at least one chimeric gene as defined above.

The subject of the invention is also a method of transforming plant cells by integration of at least one chimeric gene as defined above, which transformation may be obtained by any known appropriate means, widely described in the specialist literature and in particular the applications cited in the present application.

One series of methods consists in bombarding cells or protoplasts with particles to which the DNA sequences are attached.

Another series of methods consists in using, as means of transferring into the plant, a chimeric gene inserted into an *Agrobacterium tumefaciens* Ti plasmid or an *Agrobacterium rhizogenes* Ri plasmid.

Other methods may be used, such as microinjection or electroporation.

Persons skilled in the art will choose the appropriate method according to the nature of the plant, in particular its monocotyledonous or dicotyledonous character.

For the methods of transforming plant cells and of regenerating plants, there may be mentioned the following patents and patent applications:
US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159, EP 604 662, EP 672 752,

US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US 5,204,253, US 5,405,765, EP 442 174, EP 486 233, EP 486 234, EP 539 563, EP 674 725, WO 91/02071 and WO 95/06128.

The subject of the present invention is also the plant cells, of monocotyledonous or dicotyledonous plants, in particular of crops, which are transformed and which contain in their genome an effective quantity of a gene comprising a sequence encoding the drosomycin defined above.

The subject of the present invention is also the plants containing transformed cells, in particular the plants regenerated from the transformed cells. The regeneration is obtained by any appropriate method which depends on the nature of the species, as for example described in the above patents and applications.

The subject of the present invention is also the transformed plants derived from the cultivation and/or the crossing of the above regenerated plants, as well as the seeds of the transformed plants.

The plants thus transformed are resistant to certain diseases, in particular to certain fungal diseases. Because of this, the DNA sequence encoding drosomycin may be integrated with the main objective of producing plants resistant to the said diseases, the

drosomycin being effective against fungal diseases such as those caused by *Botrytis*, in particular *Botrytis cinerea* (mycelium or spores), *Cercospora*, in particular *Cercospora beticola*, *Septoria*, in particular *Septoria tritici*, or *Fusarium*, in particular *Fusarium culmorum* (mycelium or spores) or *Fusarium graminearum*.

The chimeric gene may also advantageously comprise at least one selectable marker, such as one or more genes for tolerance to herbicides.

The DNA sequence encoding drosomycin may also be integrated as a selectable marker during the transformation of plants with other sequences encoding other peptides or proteins of interest, such as for example genes for tolerance to herbicides.

Such genes for tolerance to herbicides are well known to persons skilled in the art and are in particular described in Patent Applications EP 115 673, WO 87/04181, EP 337 899, WO 96/38567 or WO 97/04103.

Of course, the cells and plants transformed according to the invention may comprise, in addition to the sequence encoding drosomycin, other heterologous sequences encoding proteins of interest, such as other additional peptides capable of conferring on the plant resistance to other diseases of bacterial or fungal origin, and/or other sequences encoding proteins for tolerance to herbicides and/or other sequences encoding proteins for resistance to insects, such as the *Bt* proteins in particular.

The other sequences may be integrated by means of the same vector comprising a chimeric gene, which comprises a first sequence encoding drosomycin and at least one other sequence encoding another peptide or protein of interest.

They may also be integrated by means of another vector comprising at least the said other sequence, according to the usual techniques defined above.

The plants according to the invention may also be obtained by the crossing of parents, one carrying the gene according to the invention encoding drosomycin, the other carrying a gene encoding at least one other peptide or protein of interest.

The present invention finally relates to a method of cultivating the transformed plants according to the invention, the method consisting in planting the seeds of the said transformed plants in an area of a field appropriate for the cultivation of the said plants, in applying to the said area of the said field an agrochemical composition, without substantially affecting the said seeds or the said transformed plants, and then in harvesting the cultivated plants when they reach the desired maturity and optionally in separating the seeds from the harvested plants.

Agrochemical composition is understood to mean according to the invention any agrochemical composition comprising at least one active product

having one of the following activities: herbicidal, fungicidal, bactericidal, virucidal or insecticidal activity.

According to a preferred embodiment of the method of cultivation according to the invention, the agrochemical composition comprises at least one active product having at least one fungicidal and/or bactericidal activity, more preferably exhibiting an activity complementary to that of the drosomycin produced by the transformed plants according to the invention.

Product exhibiting an activity complementary to that of drosomycin is understood to mean according to the invention a product exhibiting a complementary activity spectrum, that is to say a product which will be active against attacks by contaminants (fungi, bacteria or viruses) which are insensitive to drosomycin, or alternatively a product whose activity spectrum covers that of drosomycin, completely or in part, and whose dose for application will be substantially reduced because of the presence of the drosomycin produced by the transformed plant.

The examples below make it possible to illustrate the invention, the preparation of the chimeric gene, of the integrating vector, of the transformed plants and their resistance to various diseases of fungal origin. Figures 1 to 7 in the annex describe the schematic structures of some plasmids

prepared for the construction of the chimeric genes. In these figures, the different restriction sites are marked in *italics*.

Example 1: **Construction of the chimeric genes**

All the techniques used below are standard laboratory techniques. The detailed protocols for these techniques are in particular described in Ausubel et al.

pRPA-RD-180:

A whole cDNA clone encoding drosomycin described by Fehlbaum et al. (SEQ ID NO. 1) is inserted into the plasmid pCR-1000 (INVITROGEN) as *EcoRI-HindIII* fragment.

pRPA-RD-182: **Creation of a region encoding so-called "full-length" drosomycin (corresponding to the pre-pro peptide) by removing the nontranscribed region in 5' and by eliminating the first ATG codon.**

The plasmid pRPA-RD-180 is digested with the restriction enzymes *ScaI* and *EcoRI*, and the large DNA fragment is purified. A double-stranded synthetic oligonucleotide of the following sequence Oligo 1 is then linked to the purified DNA sequence derived from pRPA-RD-180:

Oligo 1:

5' AATTCCCGAAGACGACATGCAGATCAAGT 3'
GGGCTTCTGCTGTACGTCTAGTTCA

pRPA-RD-183: Creation of a sequence encoding mature drosomycin which does not comprise the nontranscribed region in 3'.

The two complementary synthetic oligonucleotides of sequences Oligo 2 and Oligo 3 below are hybridized at 65°C for 5 minutes and then by a slow reduction of the temperature to 30°C over 30'.

Oligo 2:

5' GAGAGATCCC CCGCGGTGGT GACTGCCTGT CCGGAAGATA
CAAGGGTCCC TGTGCCGTCT GGGACAACGA GACCTGTCGT
CGTGTGTGCA AGGAGGAGGG 3'

Oligo 3:

5' GCGCGCGGAT CCTTAGCATC CTTCGCACCA GCACTTCAGA
CTGGGGCTGC AGTGGCCACT GGAGCGTCCC TCCTCCTTGC
ACACACGACG 3'

After hybridization between Oligo 2 and Oligo 3, the DNA remaining single-stranded serves as template for the Klenow fragment of *E. coli* polymerase I (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide. This double-stranded oligonucleotide is then digested with the restriction enzymes *SacII* and *EcoRI* and cloned into the

plasmid pBS II SK(-) (Stratagene) digested with the same restriction enzymes. A clone is then obtained comprising the region encoding mature drosomycin situated between the *SacII* and *BamHI* restriction sites (SEQ ID 3).

pRPA-RD-186: Removal of the nontranscribed 3' region from the region encoding full-length drosomycin of pRPA-RD-182.

The plasmid pRPA-RD-182 is digested with the restriction enzymes *BspEI* and *KpnI*, and the large DNA fragment is purified. The plasmid pRPA-RD-183 is then digested with the restriction enzymes *BspEI* and *KpnI*, and the small DNA fragment is purified. These two purified fragments are then linked so as to obtain a plasmid containing the region encoding the pre-pro peptide of drosomycin whose first ATG codon and the two noncoding regions in 5' and 3' have been eliminated (SEQ ID 5).

pRPA-RD-187: Creation of a vector for expression in plants comprising the sequence encoding the mature form of drosomycin.

The plasmid pUGUS(118), derived from pUC-19, was obtained from Dr. Richard Vierstra of the University of Wisconsin (plasmid not described). This plasmid, whose schematic structure is represented in Figure 1, contains the CaMV 35S promoter which directs the expression of an RNA containing the untranslated sequence in 5' of the alfalfa mosaic virus (AMV 5' UTR;

Brederode et al., 1980), the N-terminal region of the *Arabidopsis thaliana* ubiquitin gene ubq11 up to the ubiquitin hydrolase cleavage site (N-term ubq11; Callis et al., 1993) which is fused in the same reading frame with the *E. coli* β-glucuronidase gene (GUS; Jefferson et al., 1987), followed by the polyadenylation site of the *Agrobacterium tumefaciens* nopaline synthase gene (NOS polyA; Bevan et al., 1983).

The plasmid pUGUS(118) is digested with the restriction enzymes *SacII* and *BamHI* and the large DNA fragment is purified. The plasmid pRPA-RD-183 is digested with the restriction enzymes *SacII* and *BamHI* and the small DNA fragment containing the region encoding the mature form of drosomycin is then purified. The two purified DNA fragments are linked together in a cassette for expression in plants which synthesize a ubiquitin-drosomycin fusion protein in the cytoplasm of plant cells. The schematic structure of this expression cassette is represented in Figure 2. Under the action of ubiquitin hydrolase on this fusion protein, the mature drosomycin is liberated into the cytoplasm of the plant cells.

<u>pRPA-RD-188</u>: Creation of a plant expression vector comprising the full length of the sequence encoding drosomycin (pre-pro).

The plasmid pRTL-2 GUS, derived from the plasmid pUC-19, was obtained from Dr. Jim Carrington (Texas A&M University, not described). This plasmid,

whose schematic structure is represented in Figure 3, contains the duplicated CaMV 35S promoter isolated from the cauliflower mosaic virus (CaMV 2×35S promoter; Odell et al., 1985) which directs the expression of an RNA containing tobacco etch virus 5' untranslated sequence (TEV 5' UTR; Carrington & Freed, 1990), the *E. coli* β-glucuronidase gene (GUS, Jefferson et al., 1987) followed by the CaMV 35S RNA polyadenylation site (CaMV polyA; Odell et al., 1985).

The plasmid pRTL-2 GUS is digested with the restriction enzymes *NcoI* and *BamHI* and the large DNA fragment is purified. The plasmid pRPA-RD-186 is digested with the restriction enzymes *BbsII* and *BamHI* and the small DNA fragment containing the region encoding drosomycin pre-pro is purified. The two purified DNA fragments are then linked together in a cassette for expression in plants which synthesize a drosomycin pre-pro protein. The schematic structure of this expression cassette is represented in Figure 4. "Pre-pro-drosomycin" represents the drosomycin coding region of pRPA-RD-186. The drosomycin is transported to the extracellular matrix of the plant by the action of a signal peptide (pre-pro).

pRPA-RD-195: Creation of a plasmid containing a modified multiple cloning site.

The plasmid pRPA-RD-195 is a plasmid derived from pUC-19 which contains a modified multiple cloning site. The complementary synthetic oligonucleotides

Oligo 4 and Oligo 5 below are hybridized and made double-stranded according to the procedure described for pRPA-RD-183.

Oligo 4: 5' AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG CATGC 3'

Oligo 5: 5' CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC TAGAGG 3'

The double-stranded oligonucleotide obtained is then linked in pUC-19 which has been previously digested with the restriction enzymes *EcoRI* and *HindIII* and made blunt-ended using the Klenow fragment of *E. coli* DNA polymerase I. A vector is obtained which contains multiple cloning sites to facilitate the introduction of the expression cassettes into an *Agrobacterium tumefaciens* vector plasmid. The schematic structure of this multiple cloning site is represented in Figure 5.

pRPA-RD-190: Introduction of the drosomycin expression cassette from pRPA-RD-187 into pRPA-RD-195.

The plasmid pRPA-RD-187 is digested with the restriction enzymes *KpnI* and *SalI*, and the DNA fragment containing the drosomycin expression cassette is purified. The purified fragment is then linked in

pRPA-RD-195 which has been previously digested with the same restriction enzymes.

pRPA-RD-191: Introduction of the drosomycin expression cassette from pRPA-RD-188 into pRPA-RD-195.

The plasmid pRPA-RD-188 is digested with the restriction enzyme *HindIII* and dephosphorylated with calf intestinal phosphatase. The DNA fragment containing the drosomycin expression cassette is purified. The purified fragment is then linked in pRPA-RP-195 which has been previously digested with the restriction enzyme *HindIII*.

pRPA-RD-174: Plasmid derived from pRPA-BL-150A (EP 0,508,909) containing the bromoxynil tolerance gene of pRPA-BL-237 (EP 0,508,909).

The bromoxynil tolerance gene is isolated from pRPA-BL-237 by PCR gene amplification. The fragment obtained is blunt-ended and is cloned into the *EcoRI* site of pRPA-BL-150A which has been made blunt-ended by the action of Klenow polymerase under standard conditions. An *Agrobacterium tumefaciens* vector is obtained which contains the bromoxynil tolerance gene near its right border, a kanamycin tolerance gene near its left border and the multiple cloning site of pUC-19 between these two genes.

The schematic structure of pRPA-RD-174 is represented in Figure 6. In this figure, "nos" represents the *Agrobacterium tumefaciens* nopaline synthase polyadenylation site (Bevan et al., 1983),

"NOS pro" represents the *Agrobacterium tumefaciens* nopaline synthase promoter (Bevan et al., 1983), "NPT II" represents the neomycin phosphotransferase gene of the *E. coli* Tn5 transposon (Rothstein et al., 1981), "35S pro" represents the 35S promoter isolated from the cauliflower mosaic virus (Odell et al., 1985), "BRX" represents the nitrilase gene isolated from *K. ozaenae* (Stalker et al., 1988), "RB" and "LB" represent respectively the right and left borders of the sequence of an *Agrobacterium tumefaciens* Ti plasmid.

pRPA-RD-184: Addition of a new unique restriction site to pRPA-RD-174.

The complementary synthetic oligonucleotides Oligo 6 and Oligo 7 below are hybridized and made double-stranded according to the procedure described for pRPA-RD-183.

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Oligo 6:  5'   CGGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC
                CCCGGCGCGC CTAGGTGTGT GCTCGAGGGC CCAACCTCAG
                TACCTGGTTC AGG  3'
Oligo 7:  5'   CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA
                CACCTAGGCG CGCCGGGGCC GCGTTTAAAC TTAATTAAGT
                GTGGCCTGAC TGG  3'
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The hybridized double-stranded oligonucleotide (95 base pairs) is purified after separation on an agarose gel (3% Nusieve, FMC). The

plasmid pRPA-RD-174 is digested with the restriction enzyme *XmaI*, and the large DNA fragment is purified. The two DNA fragments obtained are then linked.

A plasmid derived from pRPA-RD-174 is obtained which comprises other restriction sites between the bromoxynil tolerance gene and the kanamycin selectable marker gene.

The schematic structure of the plasmid pRPA-RD-184 is represented in Figure 7, where the terms "nos", "NPT II", "NOS pro", "35S pro", "BRX gene", "RB" and "LB" have the same meaning as in Figure 6.

pRPA-RD-192: Creation of an *Agrobacterium tumefaciens* vector containing the gene construct encoding drosomycin directed towards the cytosol of the cells.

The plasmid pRPA-RD-190 is digested with the restriction enzymes *ApaI* and *AscI*, and the DNA fragment containing the drosomycin expression cassette is purified. The purified DNA fragment containing the drosomycin expression cassette is linked into pRPA-RD-184, after prior digestion with the same two enzymes. An *Agrobacterium tumefaciens* vector is thus obtained which contains the sequence encoding the drosomycin-ubiquitin fusion protein which leads to the expression of drosomycin in the cytosol of the plant cells.

pRPA-RD-193: Creation of an *Agrobacterium tumefaciens* vector containing the gene construct encoding drosomycin directed towards the extracellular matrix.

The procedure described above is repeated with the plasmid pRPA-RD-191 and the restriction enzymes *PmeI* and *AscI*, replacing the plasmid pRPA-RD-190 and the restriction enzymes *ApaI* and *AscI*. An *Agrobacterium tumefaciens* vector is thus obtained which contains the sequence encoding the drosomycin pre-pro protein which leads to the expression of drosomycin in the extracellular matrix of the plant.

Example 2: Herbicide tolerance of transformed tobaccos.

2.1 - Transformation

The vectors pRPA-RD-192 and pRPA-RD-193 are introduced into the *Agrobacterium tumefaciens* EHA101 strain (Hood et al., 1987) carrying the cosmid pTVK291 (Komari et al., 1986). The transformation technique is based on the procedure of Horsh et al. (1985).

2.2 - Regeneration

The PBD6 tobacco (source SEITA France) is regenerated from foliar explants on a Murashige and Skoog (MS) basal medium comprising 30 g/l of sucrose as well as 200 µg/ml of kanamycin. The foliar explants are removed from plants cultivated in a greenhouse or *in vitro* and transformed according to the foliar disc technique (Horsh et al., 1985) in three successive stages: the first comprises the induction of shoots on a medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthyl acetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 15 days. The shoots formed

during this stage are then developed for 10 days by cultivating on an MS medium supplemented with 30 g/l of sucrose but not containing any hormone. Developed shoots are then removed and they are cultivated on an MS rooting medium containing half of the content of salts, vitamins and sugar and not containing any hormone. After about 15 days, the rooted shoots are transferred into the soil.

2.3 - Tolerance to bromoxynil

Twenty transformed plants were regenerated and transferred into a greenhouse for each construct pRPA-RD-192 and pRPA-RD-193. These plants were treated in a greenhouse at the 5-leaf stage with an aqueous Pardner suspension corresponding to 0.2 kg of bromoxynil active ingredient per hectare.

All the plants showing complete tolerance to bromoxynil are used in the following experiments to test the effects of the expression of drosomycin on the tolerance of the transformed plants to fungal attacks.

Example 3: Detection of drosomycin in transformed tobaccos

An immunoblot analysis (as described by Coligan et al.) is used to detect the drosomycin produced by the transformed tobaccos, using a rabbit antibody directed against synthetic drosomycin attached to a KLH carrier protein, with the synthetic drosomycin as antigen.

The leaf proteins are extracted, first by grinding the frozen tissues at -180°C, followed by the addition of an extraction buffer (8 M urea, 50 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% sucrose, 2 mM EDTA and 10 mM dithiothreitol). The total quantity of extractable proteins is then measured. 100 µg of extracted proteins are then loaded into SDS-PAGE gel (20% acrylamide) wells for an immunoblot analysis (according to Coligan et al.).

For the plants transformed with the plasmid pRPA-RD-193 (drosomycin pre-pro), up to 160 ng of drosomycin were found per 100 µg of total proteins extracted from the leaves.

For the plants transformed with the plasmid pRPA-RD-192 (mature drosomycin), up to 50 ng of drosomycin were found per 100 µg of total proteins extracted from the leaves.

The drosomycin synthesized and isolated from the plants transformed with the plasmids pRPA-RD-192 and pRPA-RD-193 comigrates with the drosomycin isolated from drosophila. This result show that each of the drosomycins directed either towards the cytoplasm (pRPA-RD-192) or towards the extracellular matrix (pRPA-RD-193) leads to a mature drosomycin. In addition, the gel system used in this analysis (20% acrylamide) would have made it possible to easily detect drosomycin which would not have been transformed since the two constructs (ubiquitin-drosomycin and

drosomycin pre-pro) are approximately 10 kD, against 5 kD for the mature drosomycin.

Example 4: **Resistance of the transformed tobaccos to *Botrytis cinerea***

15/20 plants derived from the plants obtained in Example 2.3 are cultivated in a greenhouse in transplanting pots of side 7 cm under the following conditions:

- temperature: 16°C at night; 19°C during the day;
- photoperiod: 14 h of darkness; 10 h of daylight;
- hygrometry: 90-1400

Two leaves per plant are inoculated with 6 discs 6 mm in diameter per leaf, each disc consisting of a *Botrytis cinerea* suspension (100,000 spores/ml). The development of the infection is observed 7 days after the inoculation by measuring the increase in the diameter of each disc.

For most of the plants transformed with the plasmid pRPA-RD-192 (mature protein in the cytoplasm) or with the plasmid pRPA-RD-193 (extracellular drosomycin), no increase in the diameters of the discs or a very small increase, is observed, which indicates a high resistance to the infections caused by *Botritis cinerea*.

Example 5: **Resistance of transformed tobaccos to *Chalara elegans***.

The preceding procedure is repeated with the following operating conditions:

- temperature: 18°C at night; 22°C during the day;
- photoperiod: 14 h of darkness; 10 h of daylight.

The inoculation is carried out 18 days after sowing by supplying to each pot 1 ml of a suspension of endoconidia containing 1,000,000 conidia/ml. The infection results are read 21 days after the inoculation by observing the roots of plantlets previously cleaned with water. The development of the disease is assessed on a marking scale from 0 to 11, 0 corresponding to an absence of infection. For the plants transformed with the plasmid pRPA-RD-192 (mature protein in the cytoplasm) and those transformed with the plasmid pRPA-RD-193 (extracellular drosomycin), the mean notation is 4, which corresponds to a high resistance to *Chalara elegans*.

The results obtained *in vivo* in Examples 4 and 5 show that the transformation with the chimeric gene according to the invention confers on the transformed plant new fungus resistance properties, an activity [lacuna] is linked to preservation of the antifungal properties of the drosomycin produced by the transformed plants according to the invention.

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CLAIMS

1. Chimeric gene comprising a coding sequence as well as heterologous regulatory elements at positions 5' and 3' capable of functioning in a plant, characterized in that the coding sequence comprises at least one DNA sequence encoding drosomycin.

2. Chimeric gene according to Claim 1, characterized in that the drosomycin essentially comprises the peptide sequence of formula (I) below:

Xaa-Cys-Xab-Cys-Xac-Cys-Xad-Cys-
Xae-Cys-Xaf-Cys-Xag-Cys-Xah-Cys (I)

in which

Xaa represents a peptide residue comprising at least 1 amino acid,

Xab represents a peptide residue of 8 amino acids,

Xac represents a peptide residue of 7 amino acids,

Xad represents a peptide residue of 3 amino acids,

Xae represents a peptide residue of 9 amino acids,

Xaf represents a peptide residue of 5 amino acids,

Xag represents a peptide residue of one amino acid, and

Xah represents a peptide residue of 2 amino acids.

3. Chimeric gene according to Claim 2, characterized in that Xab and/or Xad and/or Xac comprise at least one basic amino acid.

4. Chimeric gene according to Claim 3, characterized in that Xab comprises at least 2 basic

amino acids, preferably 2 and/or Xad and/or Xaf comprise at least 1 basic amino acid, preferably 1.

5. Chimeric gene according to either of Claims 2 and 3, characterized in that

Xaa represents the peptide sequence Xaa'-Asp- in which Xaa' represents NH_2 or a peptide residue comprising at least 1 amino acid, and/or

Xab represents the peptide sequence -Leu-Xab'-Pro- in which Xab' represents a peptide residue of 6 amino acids, and/or

Xac represents the peptide sequence -Ala-Xac'-Thr- in which Xac' represents a peptide residue of 5 amino acids, and/or

Xad represents the peptide sequence -Arg-Xad'-Val, in which Xad' represents a peptide residue of one amino acid, and/or

Xae represents the peptide sequence -Lys-Xae'-His- in which Xae' represents a peptide residue of 7 amino acids, and/or

Xaf represents the peptide sequence -Ser-Xaf'-Lys- in which Xaf' represents a peptide residue of 3 amino acids, and/or

Xag represents Trp, and/or

Xah represents the peptide residue Glu-Gly.

6. Chimeric gene according to Claim 5, characterized in that

Xab' represents the peptide sequence Ser-Gly-Arg-Tyr-Lys-Gly, and/or

Xac' represents the peptide sequence Val-Trp-Asp-Asn-Glu, and/or

Xad' represents Arg, and/or

Xae' represents the peptide sequence Glu-Glu-Gly-Arg-Ser-Ser-Gly, and/or

Xaf' represents the peptide sequence Pro-Ser-Leu.

7. Chimeric gene according to one of Claims 1 to 6, characterized in that the drosomycin is the peptide sequence represented by the sequence identifier No. 4 (SEQ ID No. 4) and the homologous peptide sequences.

8. Chimeric gene according to one of Claims 1 to 7, characterized in that the drosomycin is a "peptide-drosomycin" fusion peptide, the cutting of which by the enzymatic systems of plant cells allows the liberation of the drosomycin defined in Claims 1 to 7.

9. Chimeric gene according to Claim 8, characterized in that the "peptide-drosomycin" fusion peptide is represented by the sequence identifier No. 6 (SEQ ID No. 6).

10. "Peptide-drosomycin" fusion peptide, characterized in that the drosomycin is defined according to Claims 1 to 7.

11. Fusion peptide according to Claim 10, characterized in that the peptide is to be a signal peptide or a transit peptide.

12. Fusion peptide according to Claim 11, characterized in that the signal peptide is chosen from the signal peptide of the tobacco PR-1α gene or ubiquitin.

13. Chimeric gene according to one of Claims 1 to 12, characterized in that it comprises, in addition, at least one herbicide tolerance gene.

14. Chimeric gene according to one of Claims 1 to 14, characterized in that it comprises, in addition, at least one sequence encoding another peptide capable of conferring on the plant resistance to other diseases of bacterial or fungal origin.

15. Chimeric gene according to one of Claims 1 to 14, characterized in that the regulatory elements comprise promoter sequences, transcription activators, transit peptides and/or terminator sequences.

16. Chimeric gene according to one of Claims 1 to 14, characterized in that the regulatory promoter sequence is chosen from promoter sequences of bacterial, viral or plant origin.

17. Chimeric gene according to Claim 16, characterized in that the regulatory promoter sequence is chosen from the promoter of the gene for the ribulose-bisphosphate carboxylase/oxygenase (RuBisCO)

small subunit or that of the cauliflower mosaic (CAMV 19S or 35S).

18. Chimeric gene according to Claim 16, characterized in that the regulatory promoter sequence comprises at least one promoter chosen from histone or actin promoters.

19. Vector for transforming plants, characterized in that it contains at least one chimeric gene according to one of Claims 1 to 18.

20. Transformed plant cell containing at least one DNA as defined in one of Claims 1 to 18.

21. Disease-resistant plant, characterized in that it comprises a transformed cell according to Claim 20.

22. Plant according to Claim 21, characterized in that it is obtained by regeneration from a transformed cell according to Claim 20.

23. Disease-resistant transformed plant, characterized in that it is derived from the cultivation and/or the crossing of plants according to either of Claims 21 and 22.

24. Seeds of transformed plants according to one of Claims 21 to 23.

25. Method of transforming plants to make them resistant to diseases, characterized in that a chimeric gene according to one of Claims 1 to 18 is inserted.

26. Method of transforming plants to make them resistant to fungal diseases, characterized in that a chimeric gene according to one of Claims 1 to 18 is inserted.

27. Method according to either of Claims 25 and 26, characterized in that the transfer is carried out with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*.

28. Method according to either of Claims 25 and 26, characterized in that the transfer is carried out by supplying by bombardment with the aid of particles charged with DNA.

29. Method according to one of Claims 25 to 28, characterized in that at least one herbicide tolerance gene is also inserted.

30. Method according to one of Claims 25 to 29, characterized in that at least one sequence encoding another peptide capable of conferring on the plant resistance to other diseases of bacterial or fungal origin is also inserted.

31. Method of cultivating the transformed plants according to one of Claims 21 to 24, characterized in that it consists in planting the seeds of the said transformed plants in an area of a field appropriate for the cultivation of the said plants, in applying to the said area of the said field an agrochemical composition, without substantially

affecting the said seeds or the said transformed plants, and then in harvesting the cultivated plants when they reach the desired maturity and optionally in separating the seeds from the harvested plants.

32. Method of cultivation according to Claim 31, characterized in that the agrochemical composition comprises at least one active product having at least one fungicidal and/or bactericidal activity.

33. Method of cultivation according to Claim 24, characterized in that the active product exhibits activity complementary to that of drosomycin.

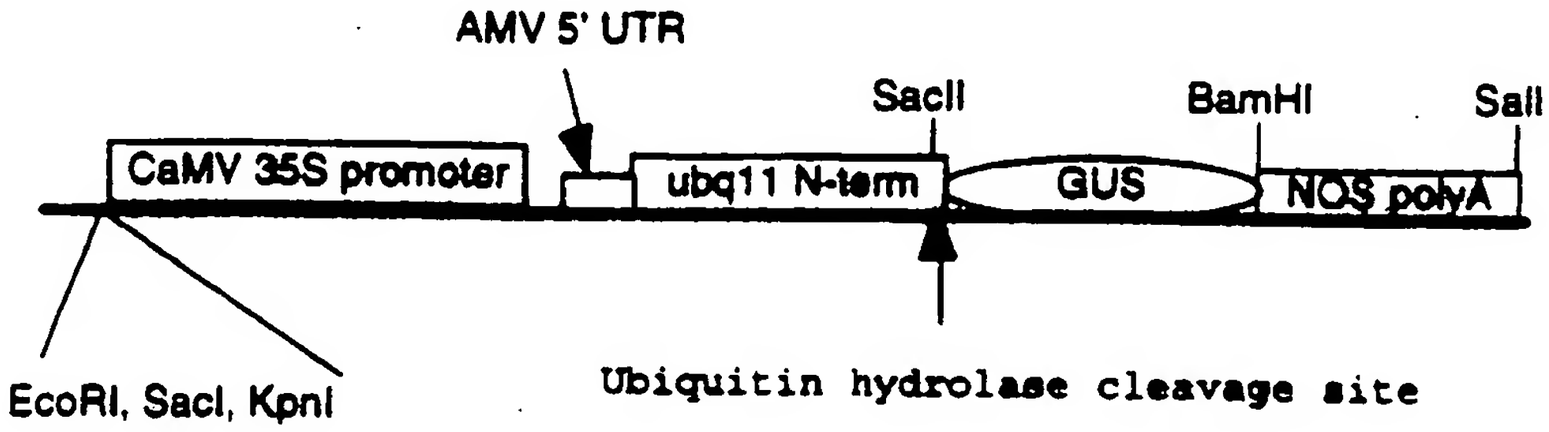

Fig. 1

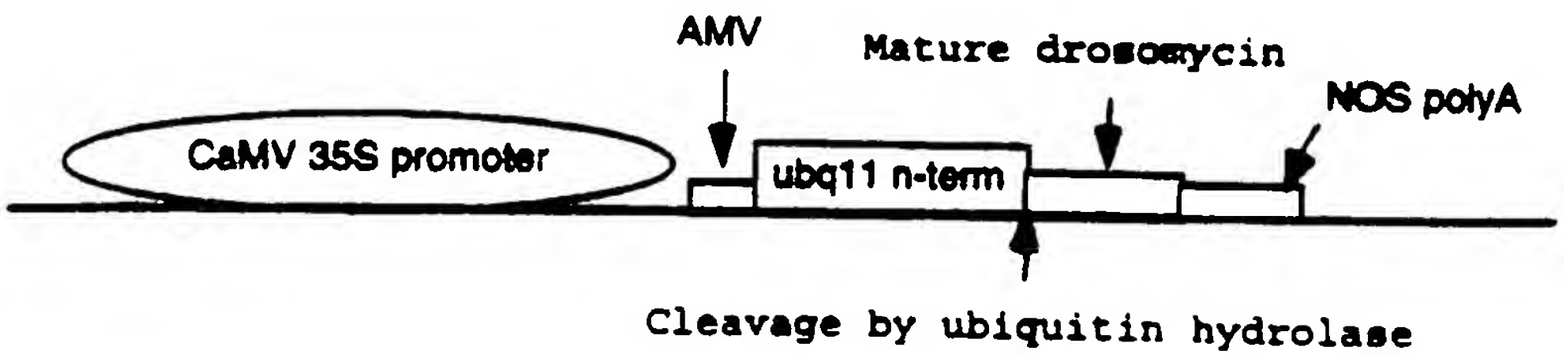

Fig. 2

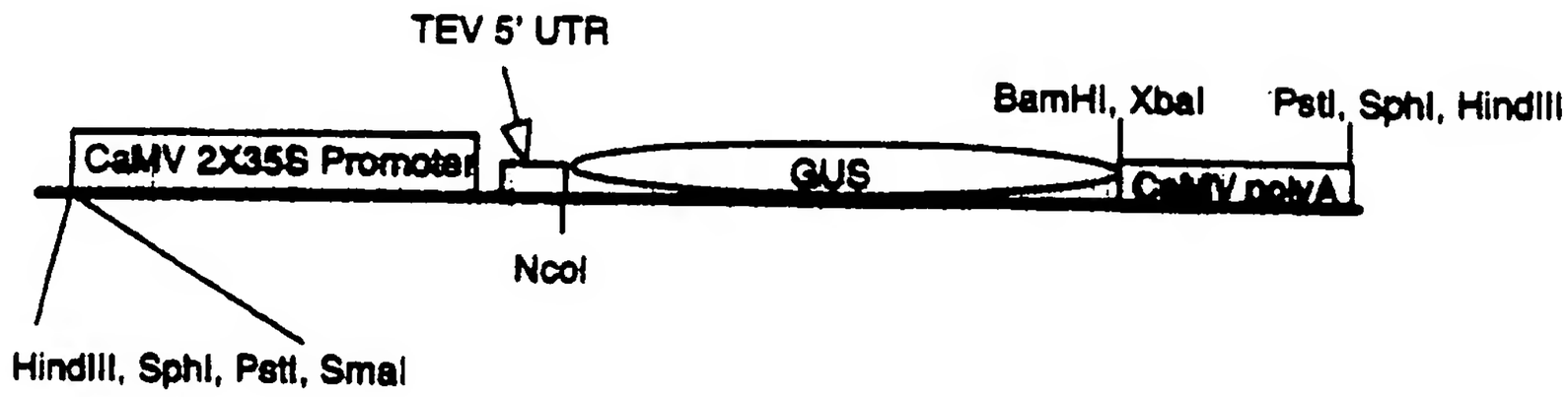

Fig. 3

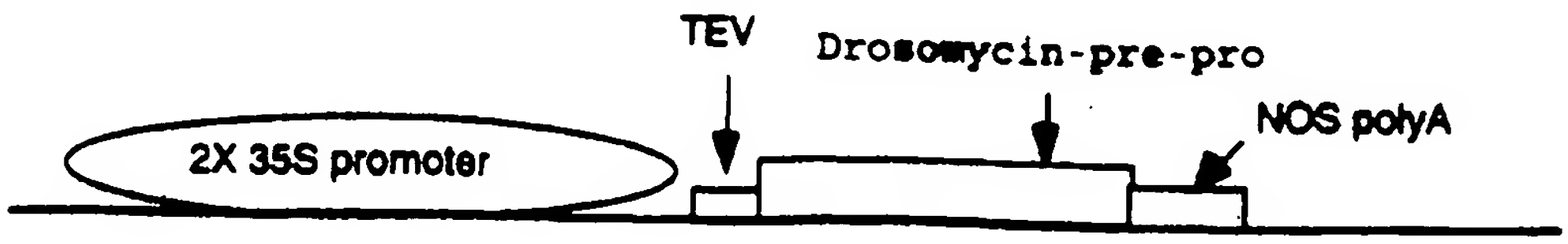

Fig. 4

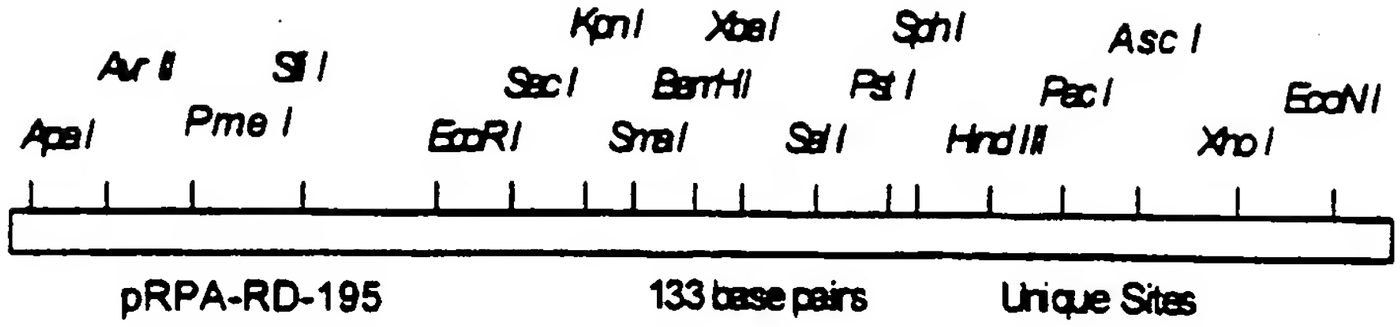

Fig. 5

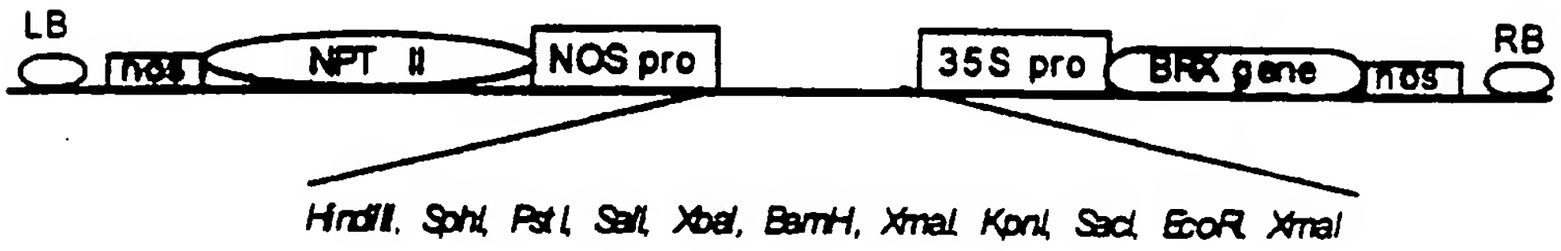

Fig. 6

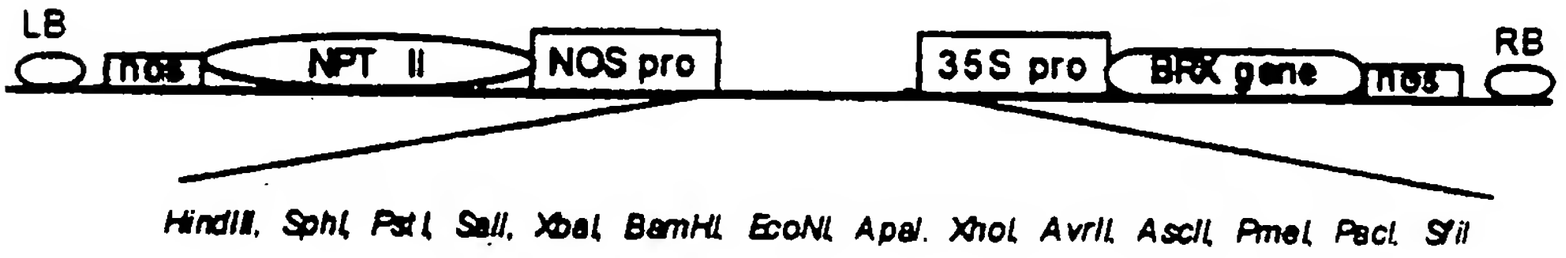

Fig. 7

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: RHONE-POULENC AGROCHIMIE

(B) STREET: 14/20 Rue Pierre BAIZET

(C) CITY: Lyon

(E) COUNTRY: France

(F) POSTAL CODE: 69009

(ii) TITLE OF INVENTION: Chimeric gene encoding drosomycin, vector containing it for the transformation of plant cells and disease-resistant transformed plants obtained

(iii) NUMBER OF SEQUENCES: 14

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 488 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Drosophila melanogaster

(vii) IMMEDIATE SOURCE:

(B) CLONE: pRPA-RD-180

(ix) ADDITIONAL FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 101..310

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1 :

```
GAATTCGAGC TCGGTACCCC TCGAGACCAT GGTCGACCCC ACGCGTCCGG ATAATTCTTT      60

CAGAAATCAT TTACCAAGCT CCGTGAGAAC CTTTTCCAAT ATG ATG CAG ATC AAG      115
                                            Met Met Gln Ile Lys
                                             1               5

TAC TTG TTC GCC CTC TTC GCT GTC CTG ATG CTG GTG GTG CTG GGA GCC      163
Tyr Leu Phe Ala Leu Phe Ala Val Leu Met Leu Val Val Leu Gly Ala
                10                  15                  20

AAC GAG GCC GAT GCC GAC TGC CTG TCC GGA AGA TAC AAG GGT CCC TGT      211
Asn Glu Ala Asp Ala Asp Cys Leu Ser Gly Arg Tyr Lys Gly Pro Cys
            25                  30                  35

GCC GTC TGG GAC AAC GAG ACC TGT CGT CGT GTG TGC AAG GAG GAG GGA      259
Ala Val Trp Asp Asn Glu Thr Cys Arg Arg Val Cys Lys Glu Glu Gly
        40                  45                  50

CGC TCC AGT GGC CAC TGC AGC CCC AGT CTG AAG TGC TGG TGC GAA GGA      307
Arg Ser Ser Gly His Cys Ser Pro Ser Leu Lys Cys Trp Cys Glu Gly
    55                  60                  65

TGC TAAATCCATG AGCAATTAGC ATGAACGTTC TGAAAAGCGC GTTTAGCTCT           360
Cys
 70

CCACTACTTA CGACATATTC TATGCTGCAA TATTGAAAAT CTAATAAACA AAACTAATGT    420

ACATTAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAGGG CGGCCGCGAC CTGCAGGCAT    480

GCAAGCTT                                                            488
```

(2) INFORMATION FOR SEQ ID NO: 2 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 70 amino acids

(B) TYPE: amino acids

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2 :

Met Met Gln Ile Lys Tyr Leu Phe Ala Leu Phe Ala Val Leu Met Leu
1 5 10 15

Val Val Leu Gly Ala Asn Glu Ala Asp Ala Asp Cys Leu Ser Gly Arg
20 25 30

Tyr Lys Gly Pro Cys Ala Val Trp Asp Asn Glu Thr Cys Arg Arg Val
35 40 45

Cys Lys Glu Glu Gly Arg Ser Ser Gly His Cys Ser Pro Ser Leu Lys
50 55 60

Cys Trp Cys Glu Gly Cys
65 70

(2) INFORMATION FOR SEQ ID NO: 3 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 167 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic

(vii) IMMEDIATE SOURCE:

(B) CLONE: pRPA-RD-183

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 21..152

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3 :

```
GAGAGATCCC CCGCCGTGGT GAC TGC CTG TCC GGA AGA TAC AAG GGT CCC          50
                      Asp Cys Leu Ser Gly Arg Tyr Lys Gly Pro
                        1               5                  10

TGT GCC GTC TGG GAC AAC GAG ACC TGT CGT CGT GTG TGC AAG GAG GAG        98
Cys Ala Val Trp Asp Asn Glu Thr Cys Arg Arg Val Cys Lys Glu Glu
                    15                  20                  25

GGA CGC TCC AGT GGC CAC TGC AGC CCC AGT CTG AAG TGC TGG TGC GAA       146
Gly Arg Ser Ser Gly His Cys Ser Pro Ser Leu Lys Cys Trp Cys Glu
                30                  35                  40

GGA TGC TAAGGATCCG CGCGC                                              167
Gly Cys
```

(2) INFORMATION FOR SEQ ID NO: 4 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4 :

```
Asp Cys Leu Ser Gly Arg Tyr Lys Gly Pro Cys Ala Val Trp Asp Asn
 1               5                  10                  15

Glu Thr Cys Arg Arg Val Cys Lys Glu Glu Gly Arg Ser Ser Gly His
            20                  25                  30

Cys Ser Pro Ser Leu Lys Cys Trp Cys Glu Gly Cys
        35                  40
```

(2) INFORMATION FOR SEQ ID NO: 5 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 236 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic

(vii) IMMEDIATE SOURCE:

(B) CLONE: pRPA-RD-186

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 15..221

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5 :

```
GAATTGAAGA CGCC ATG CAG ATC AAG TAC TTG TTC GCC CTC TTC GCT GTC        50
                Met Gln Ile Lys Tyr Leu Phe Ala Leu Phe Ala Val
                  1               5                  10

CTG ATG CTG GTG GTG CTG GGA GCC AAC GAG GCC GAT GCC GAC TGC CTG        98
Leu Met Leu Val Val Leu Gly Ala Asn Glu Ala Asp Ala Asp Cys Leu
          15                  20                  25

TCC GGA AGA TAC AAG GGT CCC TGT GCC GTC TGG GAC AAC GAG ACC TGT       146
Ser Gly Arg Tyr Lys Gly Pro Cys Ala Val Trp Asp Asn Glu Thr Cys
      30                  35                  40

CGT CGT GTG TGC AAG GAG GAG GGA CGC TCC AGT GGC CAC TGC AGC CCC       194
Arg Arg Val Cys Lys Glu Glu Gly Arg Ser Ser Gly His Cys Ser Pro
  45                  50                  55                  60

AGT CTG AAG TGC TGG TGC GAA GGA TGC TAAGGATCCG CGCGC                  236
Ser Leu Lys Cys Trp Cys Glu Gly Cys
                    65
```

(2) INFORMATION FOR SEQ ID NO: 6 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 69 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6 :

Met Gln Ile Lys Tyr Leu Phe Ala Leu Phe Ala Val Leu Met Leu Val
1 5 10 15

Val Leu Gly Ala Asn Glu Ala Asp Ala Asp Cys Leu Ser Gly Arg Tyr
20 25 30

Lys Gly Pro Cys Ala Val Trp Asp Asn Glu Thr Cys Arg Arg Val Cys
35 40 45

Lys Glu Glu Gly Arg Ser Ser Gly His Cys Ser Pro Ser Leu Lys Cys
50 55 60

Trp Cys Glu Gly Cys
65

(2) INFORMATION FOR SEQ ID NO: 7 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Synthetic Oligonucleotide 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7 :

AATTCCCGAA GACGACATGC AGATCAAGT **29**

(2) INFORMATION FOR SEQ ID NO: 8 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Synthetic Oligonucleotide 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8 :

GGGCTTCTGC TGTACGTCTA GTTCA 25

(2) INFORMATION FOR SEQ ID NO: 9 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 100 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Synthetic Oligonucleotide 2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9 :

GAGAGATCCC CGGCGGTGGT GACTGCCTGT CCGGAAGATA CAAGGGTCCC TGTGCCGTCT 60

GGGACAACGA GACCTGTCGT CGTGTGTGCA AGGAGGAGGG 100

(2) INFORMATION FOR SEQ ID NO: 10 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 100 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Synthetic Oligonucleotide 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10 :

GCGCGCGGAT CCTTAGCATC CTTCGCACCA GCACTTCAGA CTGGGGCTGC AGTGGCCACT 60

GGAGCGTCCC TCCTCCTTGC ACACACGACG 100

(2) INFORMATION FOR SEQ ID NO: 11 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 85 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Synthetic Oligonucleotide 4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11 :

AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC 60

CTCTAGAGTC GACCTGCAGG CATGC 85

(2) INFORMATION FOR SEQ ID NO: 12 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Synthetic Oligonucleotide 5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12 :

CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC 60

TAGAGG 66

(2) INFORMATION FOR SEQ ID NO: 13 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Synthetic Oligonucleotide 6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13 :

CGGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC CCCGGCGCGC CTAGGTGTGT 60

GCTCGAGGGC CCAACCTCAG TACCTGGTTC AGG 93

(2) INFORMATION FOR SEQ ID NO: 14 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Synthetic Oligonucleotide 7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14 :

CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA CACCTAGGCG CGCCGGGGCC 60

GCGTTTAAAC TTAATTAAGT GTGGCCTGAC TGG 93

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/62 C07K14/435 C12N15/12 A01H5/00
A01N63/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	P. FELHBAUM ET AL.: "Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, 1994, pages 33159-33163, XP002061373 BETHESDA, MD, US cited in the application see the whole document ---	1-11,13, 15-24, 27,29, 31-33
Y	EP 0 507 698 A (RHONE POULENC AGROCHIMIE) 7 October 1992 cited in the application see example 1 --- -/--	1-11,13, 15-24, 27,29, 31-33

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1998	03/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	FR 2 725 992 A (RHONE POULENC AGROCHIMIE) 26 April 1996 cited in the application see the whole document	1-10
A	WO 96 03522 A (DEMETER BIOTECH LTD) 8 February 1996 see page 6, line 30 - page 7, line 13	1,12,15, 19-23, 25,26
A	EP 0 508 909 A (RHONE POULENC AGROCHIMIE) 14 October 1992 cited in the application see the whole document	1,11,13, 15-17, 19-24, 27,29
A	WO 91 19738 A (HOECHST AG) 26 December 1991 see the whole document	1,19-26
A	WO 93 19188 A (MAX PLANCK GESELLSCHAFT) 30 September 1993 see page 5, line 5-37 see page 7, line 7-15	1,15,16, 19-26
A	WO 95 14098 A (BIOTECHNOLOGY RES & DEV) 26 May 1995 see abstract	1,9,10, 19
A	H. LEE ET AL.: "Structure and expression of ubiquitin genes of Drosophila melanogaster" MOLECULAR AND CELLULAR BIOLOGY, vol. 8, no. 11, 1988, pages 4727-4735, XP000644354 WASHINGTON, DC, US see the whole document	12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0507698 A	07-10-1992	FR 2673642 A	11-09-1992
		AU 652417 B	25-08-1994
		AU 1144392 A	10-09-1992
		CA 2061835 A	06-09-1992
		JP 5076369 A	30-03-1993
		MX 9200916 A	01-09-1992
		US 5491288 A	13-02-1996
		US 5792930 A	11-08-1998
FR 2725992 A	26-04-1996	NONE	
WO 9603522 A	08-02-1996	AU 3199595 A	22-02-1996
		AU 3199695 A	22-02-1996
		CA 2195625 A	08-02-1996
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		JP 10507069 T	14-07-1998
		WO 9603519 A	08-02-1996
EP 0508909 A	14-10-1992	FR 2673643 A	11-09-1992
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		US 5510471 A	23-04-1996
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		DK 535060 T	17-07-1995
		EP 0535060 A	07-04-1993
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		US 5421839 A	06-06-1995
WO 9319188 A	30-09-1993	AU 676471 B	13-03-1997
		AU 3751393 A	21-10-1993
		CA 2132323 A	30-09-1993
		EP 0631629 A	04-01-1995
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		US 5723760 A	03-03-1998
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WO 9514098 A	26-05-1995	AU 687961 B	05-03-1998
		AU 1288595 A	06-06-1995
		BR 9408093 A	12-08-1997
		CN 1136825 A	27-11-1996
		EP 0729514 A	04-09-1996
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Demande internationale No
PCT/FR 98/01462

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6 C12N15/82 C12N15/62 C07K14/435 C12N15/12 A01H5/00
A01N63/00

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 6 C12N C07K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	No des revendications visées
Y	P. FELHBAUM ET AL.,: "Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, 1994, pages 33159-33163, XP002061373 BETHESDA, MD, US cité dans la demande voir le document en entier ---	1-11,13, 15-24, 27,29, 31-33
Y	EP 0 507 698 A (RHONE POULENC AGROCHIMIE) 7 octobre 1992 cité dans la demande voir exemple 1 --- -/--	1-11,13, 15-24, 27,29, 31-33

[X] Voir la suite du cadre C pour la fin de la liste des documents

[X] Les documents de familles de brevets sont indiqués en annexe

* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

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"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée	Date d'expédition du présent rapport de recherche internationale
26 novembre 1998	03/12/1998

Nom et adresse postale de l'administration chargée de la recherche internationale	Fonctionnaire autorisé
Office Européen des Brevets, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

Formulaire PCT/ISA/210 (deuxième feuille) (juillet 1992)

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec le cas échéant, l'indication des passages pertinents	no des revendications visées
X	FR 2 725 992 A (RHONE POULENC AGROCHIMIE) 26 avril 1996 cité dans la demande voir le document en entier	1-10
A	WO 96 03522 A (DEMETER BIOTECH LTD) 8 février 1996 voir page 6, ligne 30 - page 7, ligne 13	1,12,15, 19-23, 25,26
A	EP 0 508 909 A (RHONE POULENC AGROCHIMIE) 14 octobre 1992 cité dans la demande voir le document en entier	1,11,13, 15-17, 19-24, 27,29
A	WO 91 19738 A (HOECHST AG) 26 décembre 1991 voir le document en entier	1,19-26
A	WO 93 19188 A (MAX PLANCK GESELLSCHAFT) 30 septembre 1993 voir page 5, ligne 5-37 voir page 7, ligne 7-15	1,15,16, 19-26
A	WO 95 14098 A (BIOTECHNOLOGY RES & DEV) 26 mai 1995 voir abrégé	1,9,10, 19
A	H. LEE ET AL.: "Structure and expression of ubiquitin genes of Drosophila melanogaster" MOLECULAR AND CELLULAR BIOLOGY, vol. 8, no. 11, 1988, pages 4727-4735, XP000644354 WASHINGTON, DC, US voir le document en entier	12

1

Renseignements relatifs aux membres de familles de brevets

Demande internationale No
PCT/FR 98/01462

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
EP 0507698 A	07-10-1992	FR 2673642 A	11-09-1992
		AU 652417 B	25-08-1994
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		JP 10507069 T	14-07-1998
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		ES 2071319 T	16-06-1995
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		US 5750874 A	12-05-1998
WO 9514098 A	26-05-1995	AU 687961 B	05-03-1998
		AU 1288595 A	06-06-1995
		BR 9408093 A	12-08-1997
		CN 1136825 A	27-11-1996
		EP 0729514 A	04-09-1996
		JP 9505205 T	27-05-1997